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Detection and partial characterization of an amidating enzyme in skin secretion of *Xenopus laevis*

Christa Mollay, Jitka Wichta and Günter Kreil*

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria

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Peptidyl-glycine-α-amidating monooxygenase has been purified about 150-fold from skin secretion of *Xenopus laevis* by a three-step procedure. The enzyme converts [^{14}C]succinyl-Ala-Phe-Gly to [^{14}C]succinyl-Ala-Phe-amide at both pH 5.5 and 8.4 in the presence of different metal ions and ascorbate or tetrahydropterin. In contrast to the mammalian enzyme, higher activities were detected at pH 5.5.

Posttranslational processing *α-Amidation* (*Amphibian skin*, Xenopus laevis)

1. INTRODUCTION

Numerous peptides isolated from vertebrate and invertebrate species have been found to terminate with an amide rather than a free α-carboxyl group. Without exception, the precursors of these peptides possess a glycine residue adjacent to the amino acid which is amidated in the final product [1]. As was first demonstrated by Bradbury et al. [2], the nitrogen of this glycine is retained as the terminal amide in the mature peptide. An enzyme catalyzing this amidation reaction has been isolated and partially purified from mammalian pituitary [2–4]. The enzyme has been named peptidyl-glycine-α-amidating monooxygenase (PAM) and was shown to require oxygen, ascorbate and copper ions for maximal activity [4,5].

In specialized glands of the skin of certain amphibian species, large quantities of peptides are produced which are often related to mammalian hormones and/or neurotransmitters. Many of these peptides, such as caerulein, thyrotropin releasing hormone, bombesin, physalaemin, sauvagine, dermorphin, tryptophyllins [6–8], are amidated at the carboxyl end. For the precursors of caerulein and thyrotropin releasing hormone from skin of *Xenopus laevis*, it has in fact been demonstrated, that glycine residues are present adjacent to the carboxy-terminal amino acid of the mature products [9,10]. The skin glands of *X. laevis* have been studied in some detail. These were shown to contain a syncytial secretory compartment filled with spheroidal granules [11,12]. Upon suitable stimulation, the entire content of these granules is ejected to yield a foamy secretion covering the skin of the frog. A variety of peptides as well as biogenic amines are present in the skin secretion. We asked ourselves whether the enzymes required for the processing of the precursors of these peptides might likewise be detectable in this secretion. Previously, a dipeptidyl aminopeptidase has been isolated from this secretion [13]. Here we demonstrate that it also contains PAM activity. A partial characterization of this amphibian enzyme and its cofactor requirements are presented.

2. MATERIALS AND METHODS

2.1. *Materials*

The peptide Ala-Phe-Gly was obtained from Bachem AG (Switzerland). [^{14}C]Succinic anhydride (The Radiochemical Centre, Amer-

* To whom correspondence should be addressed

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sham) was diluted with unlabeled anhydride to a specific activity of 20 μCi/μmol and incubated with Ala-Phe-Gly at pH 9 on ice. The resulting [^{14}C]succinyl-Ala-Phe-Gly was purified by high voltage paper electrophoresis at pH 4.8 (Whatman 3MM paper, 1% pyridine/acetate buffer). The radioactive peptide was eluted and used for the enzyme assays (see below). The synthetic peptide Tyr-Ala-Ala-Gly-Val-Gly-Val-Gly-Ala-Pro-Gly, which corresponds to the carboxy-terminal sequence of human calcitonin with an additional glycine, was a gift from Dr J. Leban (Biogen, Geneva).

Ascorbate was purchased from Merck (Darmstadt), tetrahydropterin from Fluka (Switzerland), bovine liver catalase from Sigma (Munich).

2.2. *Isolation of PAM from skin secretion of* X. laevis

Adult *X. laevis* were obtained from Herpetologisches Institut DeRover (The Netherlands). These were stimulated at intervals of 3 weeks by exposure to several mild electric shocks of about 15 V. The skin secretion formed within a few minutes was collected by rinsing the frogs with 0.9% NaCl. A granule fraction containing an estimated 20% of the protein of this secretion was isolated by centrifugation at 6000 rpm for 30 min. The granules were then lysed by addition of about 10 vols distilled water. Proteins in the original secretion and the lysate of the granule fraction were then precipitated by addition of ammonium sulphate. PAM activity was found to precipitate between 30 and 55% saturation. The precipitate was dissolved in 0.1 M ammonium acetate (pH 5.5) and dialyzed against this buffer at 4°C. Denatured protein was removed by centrifugation and the supernatant lyophilized. The proteins were subsequently dissolved in a small volume of the above buffer and ethanol added to a final concentration of 20%. After centrifugation, the supernatant was again lyophilized. The sample was redissolved in 0.1 M ammonium acetate, applied to a column of Affi-Gel-Con A (BioRad) and eluted with the same buffer. The eluate was lyophilized and then dissolved in buffer.

2.3. *Enzyme assay*

The partially purified enzyme preparations were incubated with [^{14}C]succinyl-Ala-Phe-Gly at 36°C for periods of up to 3 h. The product [^{14}C]succinyl-Ala-Phe-amide could be separated from the substrate by high voltage paper electrophoresis at pH 4.8. While the latter migrates with a mobility relative to free glutamic acid of about 1.1, the product migrates at about 0.55 relative to Glu. Dried pherograms were cut into sections and the radioactivity present on each was determined in a liquid scintillation counter (toluene based scintillation fluid). From the known specific activity and the counting efficiency determined for the conditions used for the assays, 300 cpm were estimated to correspond to 1 pmol succinyl-peptide. The unlabeled calcitonin fragment was also separated from the reaction product by paper electrophoresis at pH 4.8 and the peptides were stained with ninhydrin.

3. RESULTS AND DISCUSSION

Crude skin secretion of *X. laevis* was found to contain an enzymatic activity which converts succinyl-Ala-Phe-Gly to succinyl-Ala-Phe-amide. The reaction was stimulated up to 5-fold by the addition of copper sulphate and ascorbate. The extent of this stimulation was quite variable between different preparations of skin secretion.

Using ammonium sulphate fractionation, ethanol precipitation and chromatography on Affi-Gel-Con A (see section 2.2), a partially purified enzyme preparation was obtained. Using the granule fraction collected from skin secretion as the starting material, PAM activity could be purified about 150-fold by this procedure. Separation of the partially purified enzyme by SDS-polyacrylamide gel electrophoresis and subsequent staining with Coomassie blue still revealed the presence of several proteins (see fig.1). However, two proteins with M_r values of about 54000 and 58000 were enriched in the most active fractions and one of these may thus be the amidating enzyme. The partially purified enzyme could be frozen and thawed or lyophilized without an appreciable loss of activity. Besides the radioactive peptide mentioned above, the enzyme preparation was also incubated with the peptide Tyr-Ala-Ala-Gly-Val-Gly-Val-Gly-Ala-Pro-Gly overnight at 36°C and pH 5.5. After separation by high voltage paper electrophoresis, the corresponding amidated

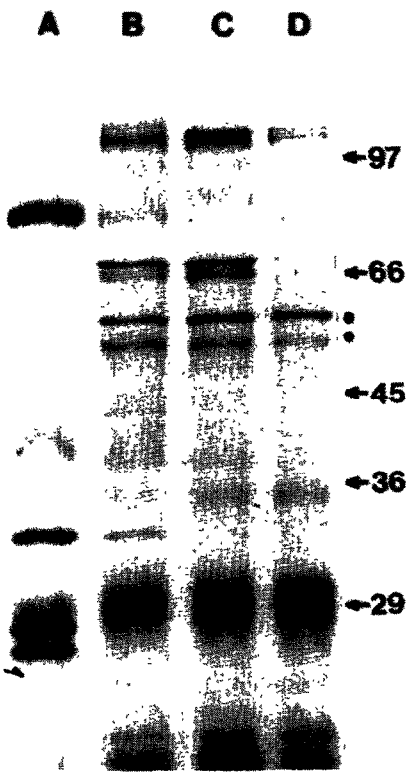

Fig.1. Analysis of different fractions by gel electrophoresis in 10% SDS-polyacrylamide. (A) Granule fraction of skin secretion; (B) proteins precipitating between 30 and 55% saturated ammonium sulphate; (C) supernatant after precipitation with 20% ethanol; (D) eluate from Affi-Gel-Con A. Numbers refer to molecular masses of standards (kDa). The proteins with molecular masses of about 54 and 58 kDa are marked by dots.

Table 1

Activity of PAM from skin of *X. laevis* at different pH and in the presence of different cofactors

Additions	pH 5.5	pH 8.4
None	540	460
Fe^{3+}, tetrahydropterin	3950	1095
Cu^{2+}, tetrahydropterin	2950	2040
Fe^{3+}, ascorbate	2260	1850
Cu^{2+}, ascorbate	820	2000

The partially purified enzyme was incubated at 36°C in the presence of metal ions and cofactors at the following concentrations: 2 mM ascorbate, 2 μM Cu^{2+}, 2 μM Fe^{3+}, 3.5 mM tetrahydropterin. All assays also contained 0.1 mg/ml of catalase. The formation of [^{14}C]succinyl-Ala-Phe-amide from [^{14}C]succinyl-Ala-Phe-Gly is given in pmol/mg per h

Table 2

Activity of PAM in the presence of different metal ions

Metal	pmol/mg per h
None	635
Mn^{2+}	3240
Fe^{3+}	2780
Zn^{2+}	1990
Fe^{2+}	1820
Cu^{2+}	1550

Samples were incubated at 36°C in 0.1 M ammonium acetate, pH 5.5 in the presence of 3.5 mM tetrahydropterin, 0.1 mg/ml catalase and 2 μM metal ion. Yields are expressed as in table 1

peptide could be detected by staining with ninhydrin (not shown).

Enzymatic tests with the partially purified enzyme preparations have shown that the cofactor requirement of the frog skin enzyme is apparently different from the one found for the mammalian PAM. At pH 8–9, the frog skin enzyme was most active with copper ions and ascorbate, as has been shown for the mammalian enzyme [4,5]. However, at pH 5.5, higher reaction rates were observed in the presence of ferric ions and tetrahydropterin (see table 1). Moreover, even after the different purification steps, the frog skin enzyme still showed activity in the absence of any added cofactors. This indicates that the natural cofactors, which are currently not known, are fairly tightly bound to the protein. In table 2, we show the activity of the frog skin enzyme in the presence of tetrahydropterin and different metal ions at pH 5.5. Under these conditions, up to 2-times higher reaction rates were observed in the presence of ferric or manganese ions as compared to copper ions.

These results demonstrate the presence of PAM in skin secretion of *X. laevis*. The frog enzyme appears to be more stable than the corresponding mammalian enzyme and it may also differ in its cofactor requirements. We are currently collecting larger quantities of this skin secretion to purify the enzyme to homogeneity and study its properties in more detail.

It is intriguing that frog skin secretion contains at least two enzymes involved in the processing of peptide precursors. These are a dipeptidyl aminopeptidase [13] and, as shown here, the enzyme catalyzing the formation of carboxy-terminal amides. Further studies will be aimed at establishing whether other processing enzymes, like the endoproteases specifically cleaving after pairs of basic or single arginine residues, coexist in the skin exudate. Earlier attempts to purify these enzymes from animal cells have been plagued by artefacts due to contaminations with lysosomal and other proteases. This difficulty may not exist in the case of amphibian skin secretion.

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